Application of a cDNA-AFLP approach to study pea resistance to *Didymella pinodes* in *Pisum fulvum* at molecular level

Nicolas Rispail[1], Sara Fondevilla[1,2], Diego Rubiales[1]

[1]Institute for Sustainable Agriculture – CSIC, Alameda del Obispo, Avda. Menendez Pidal s/n, 14080 Cordoba, Spain. [2] University of Frankfurt, Germany

Introduction

Didymella pinodes (formerly *Mycosphaerella pinodes*) is the most predominant and damaging causal agent of the Ascochyta blight disease of pea (*Pisum sativum* ssp. *sativum*). So far, no complete resistance to this pathogen has been described in pea. The highest levels of incomplete resistance have been identified in wild relatives of pea. A recent screening of a collection of wild pea relative identified several wild accessions with incomplete resistance. Among them, the *P. fulvum* accession P651 was the most resistant (1). This accession showed both smaller and fewer lesion than susceptible check Messire (1). At cellular level, the resistance of this accessions was associated to a higher production of hydrogen peroxide and to protein cross-linking at attack site which create a physical and chemical barrier that limit the development of the pathogen (2). Identification of the genes controlling resistance to *D. pinodes* in this wild resistant accession would facilitate their introgression into pea varieties. Thus we targeted the molecular characterization of resistance mechanism in this accessions.

Methods

Since little information is available on *P. fulvum* genome, a cDNA-AFLP approach was chosen to identify genes specifically involved in the defence/resistance reaction of this highly resistant accessions P651 by comparison with a susceptible cultivar Messire. For this, leaf samples of the resistant P651 and susceptible Messire accessions were collected after 24 and 48 hours post-inoculation with *D. pinodes*. Control samples maintained non inoculated were also harvested. After extraction of Total RNA with Trisure (Bioline), the samples were transformed in two-stranded cDNA with the Superscript cDNA synthesis kit (Invitrogen) followed by treatment with the DNA Polymerase I (Promega). The gene profiles were then obtained following the method described by Bachem et al., (3) after sample digestion with EcoRI and MseI and selectively amplified with primers containing 1 and 3 selective nucleotide respectively. Transcribed differential fragments (TDFs) were separated on 4.5% polyacrylamide gel and visualized by silver staining. Interesting TDFs were then excised from the gel, re-amplified with the same primer pairs, cloned and sequenced.

Data Summary:

- **Primer combination used:** 30
- **Total number of Band:** 890
- **Mean Band/Profile:** 52
- **Total number of TDFs:** 425 (48%)

- **Excised TDFs:** 172 (from 15 profiles)
- **Re-amplified TDFs:** 51 (from 9 profiles)
- **Sequenced TDFs:** 19

Results

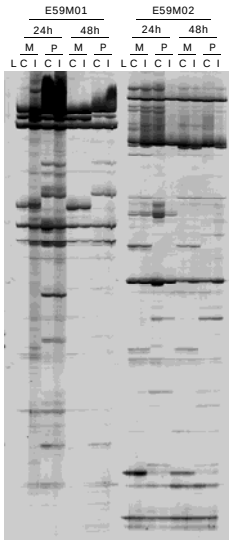

Figure 1. Example of gene profiles.

Sequential amplification of the pool of differential fragments with primer containing 1 and 3 selecting nucleotides respectively allow establishing several gene profile as shown in Fig. 1. Comparison of the profiles obtained so far for the different samples allowed identifying many transcribed differential fragments (TDFs) between treatment, time of sampling and accessions (see data summary section). Interestingly most of these TDFs were accession specifics confirming the high genetic distance existing between both accessions. These differential fragments are unlikely related to the defence reaction but may be very useful molecular markers for future mapping studies (see insert below). Thus all TDFs are being excised from gels for further analysis.

To date only a subset of the TDFs have been already re-amplified and sequenced (see Data summary). These TDFs were then further analysed by BLAST searches using the Genbank and *M. truncatula* v3.5 genome information databases in the NCBI and JCVI server respectively. Nearly all sequences could be identified with only one sequence that did not match to any known sequences in the databases (E07M09_37). While two sequences (E23M02_44 and E30M01_33) only match to *P. sativum* ESTs with no known function, all the others matched to annotated *M. truncatula* genes of different cellular processes. Interestingly, several of them were directly related to defence reaction including the monodehydroascorbate reductase (E23M02_36) or matched with ESTs isolated within biotic and abiotic legume libraries.

Table 1. Annotation of Sequenced TDFs according to BLAST searches

Band	Best BLAST Hit	BLAST Score	Evalue	Anotation
E07M09_28	Medtr4g085890.1 3-methylcrotonyl-CoA carboxylase	1057	$7.3e^{-42}$	3-methylcrotonyl-CoA carboxylase
E07M09_31	Medtr4g085890.1 3-methylcrotonyl-CoA carboxylase	923	$9.3e^{-36}$	3-methylcrotonyl-CoA carboxylase
E07M09_32	Medtr1g100680.1 Methionine synthase	872	$2.9e^{-37}$	Methionine synthase
E07M09_37	No hit			Unknown sequence
E07M09_38	contig_53428_1.1 V-type proton ATPase subunit G	427	$3.5e^{-15}$	V-type proton ATPase subunit G
E23M02_10	Medtr6g023910.1 Short-chain alcohol dehydrogenase	682	$2.8e^{-41}$	Short-chain alcohol dehydrogenase
E23M02_16	*P. sativum* magnesium chelatase H subunit (CHLH)	568	$1e^{-158}$	chloroplast magnesium chelatase H subunit
E23M02_17	Medtr6g031100.1 hypothetical protein	740	$1.9e^{-27}$	hypothetical protein
E23M02_24	Medtr8g022310.1 Chaperone protein dnaJ	345	$1.8e^{-09}$	Chaperone protein dnaJ
E23M02_36	contig_237706_1.1 Monodehydroascorbate reductase	527	$9.2e^{-19}$	Monodehydroascorbate reductase
E23M02_44	TSA: Ps_contig_mira-ass_23823 mRNA	285	$1e^{-74}$	Unknow sequence
E30M01_21	Medtr5g051180.1 hypothetical protein	1288	$2.8e^{-53}$	26S ribosomic protein
E30M01_23	contig_168999_1.1 Cystathionine gamma-synthase	957	$5.5e^{-38}$	Cystathionine gamma-synthase
E30M01_33	TSA: Ps_contig_mira-ass_5946 mRNA	267	$6e^{-69}$	Unknow sequence
E30M02_22	Medtr3g104850.1 Omega-amidase NIT2	348	$1.1e^{-21}$	Omega-amidase NIT2
E30M02_30	Medtr3g107520.1 Cellulose synthase	418	$9.5e^{-13}$	Cellulose synthase
E30M02_31	*P. sativum* putative malate dehydrogenase (MDH)	331	$1e^{-87}$	Malate dehydrogenase
E39M01_09	Medtr7g084590.1 Syntaxin-22	753	$3.4e^{-52}$	Syntaxin-22

Concluding Remarks:

- Several novel pea genes have been identified
- Some of them may play a role in pea defence against *D. pinodes* and will be studied further
- Novel polymorphic marker are being generated

Characterisation of molecular markers

Among the sequenced gene fragments, two TDFs, E07M09_28 and E07M09_31, appear to encode for the same gene (Table 1). Pair-wise alignment of these sequences specifically expressed in P651 and Messire accession, respectively, showed a very high level of conservation. Both sequences shared more than 99% identity except for the presence of a small insert of 12 nt at 3' extremity of the gene fragment in the *P. fulvum* sequence which is absent in the *P. sativum* cultivar Messire. Further analysis in legume genomic databases indicated that this insertion was detected in sequence orthologs of most legume species including lentils, faba bean and *Medicago truncatula* but not in the *P. sativum* sequences. Thus this sequence would be a good candidate to design a SCAR marker for future interspecific mapping program.

Figure 2. Pair-wise alignment between the E07M09_28 and E07M09_31.
Alignment was obtained using the needle algorithm implemented in the EMBOSS server.

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E07M09B31        1 AGTGGAACAAGGAGGAGGAAGAAAAGTTCAAAAGAAAAGTAGTTGAGGCG     50
                   |||||||||||||||||||||||||||||||||||||||||||||||||||
E07M09B28        1 AGTGGAACAAGGAGGAGGAAGAAAAGTTCAAAAGAAAAGTAGTTGAGGCG     50

E07M09B31       51 TACGAGAGAGAAGGAAGTCCTTATTACTCAACGGCGAGGCTTTGGGATGA    100
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||
E07M09B28       51 TACGAGAGAGAAGGAAGTCCTTATTACTCAACGGCGAGGCTTTGGGATGA    100

E07M09B31      101 TGGAATTATTGATCCAGCTGATACAAGAAAAATAATTGGTCTATGTGTTT    150
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||
E07M09B28      101 TGGAATTATTGATCCAGCTGATACAAGAAAAATAATTGGTCTATGTGTTT    150

E07M09B31      151 CAGCTTCCTTGAACCGTCCCATAGAAAATACCAAATATGGTGTATTTAGA    200
                   ||||||||||||||||||||.|||||||||||||||||||||||||||||
E07M09B28      151 CAGCTTCCTTGAACCGTCCCACAGAAAATACCAAATATGGTGTATTTAGA    200

E07M09B31      201 ATG--------------TGAAGCTTTGTTGTGTGCTAAATGTTG    230
                   |||              |||||||||||||||||||||||||
E07M09B28      201 ATGTGAATAAGTGATGTTGAAGCTTTGTTGTGTGCTAAATGT--    242
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